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Inducement and Reversal of Tetracycline Resistance in *Escherichia coli* K-12 and Expression of Proton Gradient-Dependent Multidrug Efflux Pump Genes

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Expression of eight transporter genes of *Escherichia coli* K-12 and its Δ*acrAB* mutant prior to and after induction of both strains to tetracycline resistance and after reversal of induced resistance were analyzed by quantitative reverse transcriptase PCR. All transporter genes were overexpressed after induced resistance with *acrF* being 80-fold more expressed in the Δ*acrAB* tetracycline-induced strain.

An organism responds to noxious agents present in its environment by altering the level of expression of those genes that favor survival and continued growth. Natural intrinsic resistance to these agents in gram-negative bacteria, that is, resistance not associated with any chromosomal mutation or the acquisition of extrachromosomal elements with resistance determinants, can be increased by preventing the antibiotic from entering the cell through the control of the permeability and by the effectiveness of efflux pumps present in the cell envelope that extrude one or more of these agents (15, 17, 21, 24). The permeability barrier alone does not produce significant antibiotic resistance because mutants with decreased expression of specific porins show only small increases in antibiotic resistance (17, 23, 24). Efflux transporters exist in limited numbers with fixed activity and accommodate the extrusion of these agents up to certain limits (15, 21). When the concentration exceeds the capacity of the pumps, the organism is at risk for survival. *Escherichia coli* has been shown to have at least nine intrinsic major different proton-dependent multidrug-resistant efflux pump systems (MDR pumps) that bestow resistance to two or more of these antimicrobial agents (15, 28). The genes coding for each of these efflux pumps are *emrE* (30), *acrEF* (formerly *envCD*) (12, 13, 31), *emrAB* (16), *emrD* (20), *acrAB*-TolC (8, 18, 19), *mdfA* (formerly *cmr*) (6, 25), *tehA* (35), *acrD* (an *acrB* homolog) (32), and *yhiV* (26). They belong to one of three genetically and structurally defined families: the major facilitator superfamily (MFS; *emrD*, *mdfA*, *emrB*), the resistance nodulation-cell division family (RND; *acrB*, *acrF*, *acrD*, *yhiV*), and the small multidrug resistance family (SMR; *emrE*, *tehA*) (15, 28).

The tripartite AcrAB-TolC system is the most well studied MDR pump system and consists of an inner-membrane efflux transporter (AcrB) that removes a large gamut of nonrelated antibiotics from the cytoplasm into the periplasm, where the linker protein (AcrA) directs the intermembrane transport of the antibiotic through the outer membrane channel (TolC) to the outside (8, 22). This type of genetic and structural organization is shared with its analog AcrEF, and their role has been assumed from the demonstration that *acrAB* or *acrEF* mutants are increasingly susceptible to a wide variety of antibiotics and detergents (12, 27, 34) and that these deletion mutants could be made significantly more resistant to these substances by the respective insertion of *acrAB*- or *acrEF*-carrying plasmids (2, 15, 26, 28). Although *E. coli* has been shown to have these intrinsic proton-dependent multidrug-resistant efflux pump systems, the specific activity of any one pump in its natural state and level has not yet been reported.

Bacteria initially susceptible to antibiotics have been made resistant by stepwise exposure to these compounds, and this resistance could be reversed by serial transfer to drug-free medium (9) or by exposure to inhibitors of efflux pumps (29, 36). The use of an *E. coli* K-12 strain whose genes that code for the main efflux pump AcrAB have been deleted (AG100A), together with its parental wild-type AG100 (9, 27), provided us the opportunity to study the expression, with the aid of the quantitative real-time reverse transcriptase PCR (RT-PCR) (Table 1), of the nine multidrug-resistant efflux pump systems of *E. coli*, prior to and after inducing resistance to tetracycline by slow and gradual exposure to the antibiotic, and after the strains have reverted to the original tetracycline susceptibility of their respective parents.

The tetracycline-susceptible *E. coli* AG100 parent strain (MIC of 2.0 mg/liter determined by the broth macrodilution method) (7, 27) and its AG100A Δ*acrAB* insertion mutant progeny that is four times more susceptible to the antibiotic (MIC, 0.5 mg/liter) (Table 2) were induced by gradual stepwise increase of tetracycline to significant levels of resistance to the antibiotic (Fig. 1). Repeated serial transfer of these tetracycline-induced resistant strains to drug-free medium eventually restored the level of susceptibility to that initially present in the

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TABLE 1. Primers and conditions used in this study[a]

Efflux transporter and housekeeping gene	Primer sequence (5′–3′)[b]	Length of amplicon (bp)
acrB	CGTACACAGAAAGTGCTCAA/CGCTTCAACTTTGTTTTCTT	183
acrD	GATTATCTTAGCCGCTTCAA/CAATGGAGGCTTTAACAAAC	187
acrF	TAGCAATTTCCTTTGTGGTT/CCTTTACCCTCTTTCTCCAT	247
emrB	ATTATGTATGCCGTCTGCTT/TTCGCGTAAAGTTAGAGAGG	196
emrD	TGTTAAACATGGGGATTCTC/TCAGCATCAGCAAATAACAG	243
emrE	GGATTGCTTATGCTATCTGG/GTGTGCTTCGTGACAATAAA	156
mdfA	TTTATGCTTTCGGTATTGGT/GAGATTAAACAGTCCGTTGC	182
tehA	TGCTTCATTCTGGAGTTTCT/TCATTCTTTGTCCTCTGCTT	232
yhiV	GCACTCTATGAGAGCTGGTC/CCTTCTTTCTGCATCATCTC	203
GAPDH	ACTTACGAGCAGATCAAAGC/AGTTTCACGAAGTTGTCGTT	170

[a] Amplification performed in separate tubes using the same amount of total RNA retrieved from the same sample. Thermal cycling conditions: reverse transcription at 50°C/30 min, PCR activation at 95°C/15 min, followed by 35 cycles of denaturation (94°C/60 s), annealing (51°C to 53°C/60 s depending on primers used), and extension (72°C/60 s). Primers were designed based on the sequence for the *E. coli* K-12 complete genome (accession number NC_000913) (1).
[b] Forward primer sequences appear before the slash and reverse primer sequences appear after the slash.

AG100 and AG100A strains after 40 and 110 days, respectively (Fig. 1).

The relative quantities of the inner-membrane efflux transporter gene mRNA, isolated with the aid of an RNeasy Protect Mini Kit (QIAGEN, Hilden, Germany), of each of the nine major *E. coli* proton-dependent efflux pump systems were determined by the comparative threshold cycle (C_T) method (14, 33) in a Rotor-Gene 2000 thermocycler with real-time analysis software (Corbett Research, Sydney, Australia) using a QuantiTect SYBR Green RT-PCR Kit (QIAGEN). The levels expressed by the wild-type *E. coli* AG100 and its Δ*acrAB* mutant prior to and after tetracycline resistance had been induced and reversed were determined for cultures in the presence of tetracycline concentrations immediately below the MIC for each strain. Firstly, direct comparison of the expression levels of the genes that code for the transporter proteins between *E. coli* AG100 and AG100A prior to exposure to tetracycline, with the exception of *acrB* which is not expressed in the Δ*acrAB* mutant, showed that the expression of the remaining eight genes is practically identical (data not shown). The relative expression levels of the transporter genes, after prolonged serial exposure of *E. coli* AG100 and AG100A strains to increasing concentrations of tetracycline and after reversal of resistance by transfer to drug-free medium ($AG100_{REV}$ and $AG100A_{REV}$), are presented in Fig. 2. Tetracycline resistance induced in the wild-type AG100 strain ($AG100_{TET}$) as well as in the Δ*acrAB* mutant ($AG100A_{TET}$) results in the increased expression of all the efflux transporter genes over that expressed by either respective parental strain, an increase substantially higher in the deleted mutant (Fig. 2A and B). It is important to note that the *acrF* gene of the $AG100A_{TET}$ strain is markedly overexpressed (80-fold increase relative to the noninduced AG100A; Fig. 2B). Transfer of the induced tetracycline-resistant strains to

TABLE 2. MIC values of antibiotics and other agents known to be substrates of efflux pumps against *E. coli* AG100, AG100A (*acrAB* deleted), $AG100_{TET}$, and $AG100A_{TET}$[a]

Agent	MIC (mg/liter)			
	AG100	AG100A	$AG100_{TET}$	$AG100A_{TET}$
Antibiotic				
TET	2.0	0.5	12	12
KAN	15	>200	10	>200
ERY	100	6.25	100	100
OFX	0.12	0.015	0.48	0.48
CIP	0.03	0.004	0.12	0.12
CHL	8	2	>16	>16
PEN	16	8	64	32
OXA	256	1	>512	>512
Substrate				
SDS	>800	50	>800	>800
RHO	>800	100	>800	>800
TPP	1,500	15	2,000	1,500
BC	15	2	15	15
EB	150	5	>200	>200

[a] *E. coli* $AG100_{TET}$ and $AG100A_{TET}$ induced to tetracycline resistance by serial gradual and stepwise increase of tetracycline concentration as described in the text. Antibiotics: tetracycline (TET), kanamycin (KAN), erythromycin (ERY), ofloxacin (OFX), ciprofloxacin (CIP), chloramphenicol (CHL), penicillin (PEN), and oxacillin (OXA). Efflux pump substrates: sodium dodecyl sulfate (SDS), rhodamine (RHO), tetraphenylphosphodium bromide (TPP), benzalkonium chloride (BC), and ethidium bromide (EB).

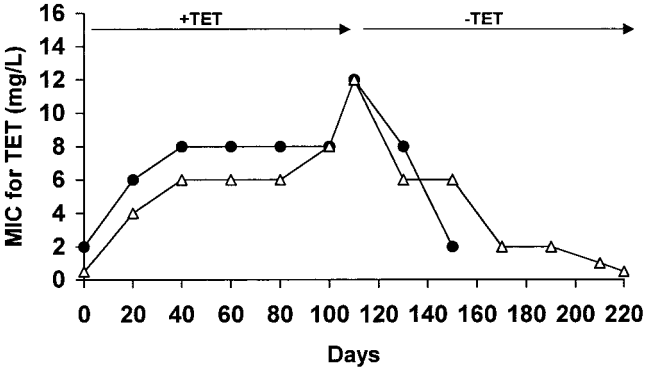

FIG. 1. Time course of induced tetracycline resistance of *E. coli* AG100 (solid circles) and AG100A (open triangles) strains and the reversal of induced resistance by transfer to drug-free medium. Strains were induced to tetracycline resistance (+TET) by serial transfer of inoculae from cultures that contained the highest concentration of the antibiotic under which the strains grew to media containing increasing concentrations of the drug and incubated until they yielded prominent evidence of growth. Reversal of resistance was induced by serial transfer to drug-free medium (-TET). MICs were periodically determined and confirmed in solid media by the tetracycline E-test (AB Biodisk, VIVA Diagnostica, Huerth, Germany) according to the manufacturer's instructions.

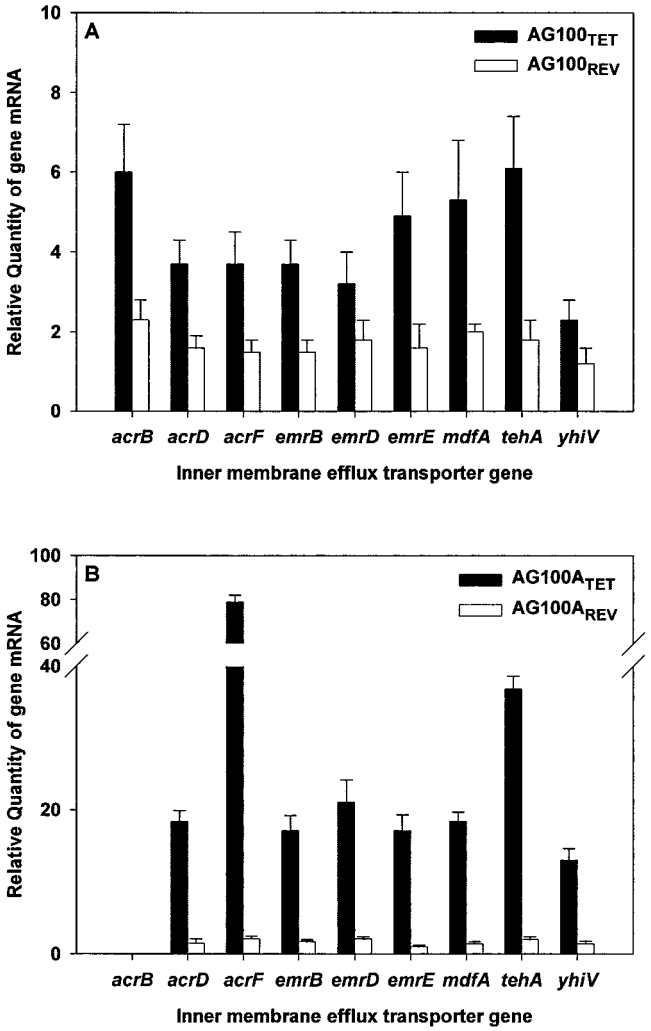

FIG. 2. Quantification of the expression level of the inner membrane efflux transporter genes of the nine major *E. coli* proton-dependent efflux pump systems in the tetracycline-induced resistant $AG100_{TET}$ and $AG100A_{TET}$ and the two strains after reversion of tetracycline resistance ($AG100_{REV}$ and $AG100A_{REV}$,) relative to wild-type AG100 and the *acrAB*-deleted progeny AG100A, respectively. Gene transcript levels were normalized against the *E. coli* housekeeping gene *GAPDH* (coding for D-glyceraldehyde-3-phosphate dehydrogenase) (3) measured in the same sample. Data are from three independent total mRNA extractions with corrections for standard deviation range.

drug-free medium eventually restored the expression of the inner-membrane efflux transporter genes to that of their respective noninduced parents (Fig. 2). However, restoration of susceptibility took place much faster for the wild-type AG100 (40 days) (Fig. 1).

The susceptibility of strains AG100, AG100A, $AG100_{TET}$, and $AG100A_{TET}$ to a panel of antibiotics is described in Table 2. Briefly, with the exception of kanamycin (resistance insertion marker, Δ*acrA*B::Tn*903* Kan^r [27]), susceptibility of AG100A to all of the antibiotics of the panel is significantly greater than that demonstrated by the parental AG100 strain, confirming the studies of others (11, 27). The AG100A is also far more susceptible to sodium dodecyl sulfate, rhodamine 6G,

TABLE 3. MIC values for inhibitors of efflux pumps against AG100, AG100A (*acrAB* deleted), $AG100_{TET}$, and $AG100A_{TET}$[a]

Inhibitors	MIC (mg/liter)			
	AG100	AG100A	$AG100_{TET}$	$AG100A_{TET}$
Calcium channel				
CPZ	60	20	140	120
TZ	100	25	>200	100
RES	140	140	100	100
VP	>3,000	450	>3,000	>3,000
Proton pumps				
OM	>2,500	>2,500	>2,500	>2,500
CCCP	10	10	20	20
PAN	>200	50	>200	>200

[a] The maximum solubility in the medium for some of the agents was reached with no effect on growth. Hence the MIC was arbitrarily identified as "greater than." Calcium channel inhibitors: chlorpromazine (CPZ), thioridazine (TZ), reserpine (RES), and verapamil (VP). Proton pump inhibitors: omeprazole (OM), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), and Phe-Arg-napthylamide (MC-207,110) (PAN).

tetraphenilphosphonium (TPP), benzalkonium chloride (BC), and ethidium bromide (EB), all of which have been identified as substrates of the AcrAB-TolC pump system (34). EB, TPP, and BC are also substrates of the MdfA (6), the EmrE (18, 30), and the TehA (35) MDR pump systems. The susceptibility of tetracycline-induced resistant strains $AG100_{TET}$ and $AG100A_{TET}$ to the panel of antibiotics tested, including β-lactams, with the exception of kanamycin, is significantly decreased, as shown by the data presented in Table 2. As a consequence of inducing tetracycline resistance, the initial differences in antibiotic susceptibility between AG100 and AG100A strains are essentially lost. It is important to note that the marked initial susceptibility of AG100A to the five efflux pump substrates tested is also lost subsequent to the inducing of tetracycline resistance (see Table 2).

Similarly, with respect to the effects of known inhibitors of bacterial efflux pumps on the growth of AG100, AG100A, and their tetracycline-induced resistant progeny, with the exception of reserpine, omeprazole, and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), AG100A is significantly more susceptible to verapamil (VP), Phe-Arg-napthylamide (MC-207,110) (PAN), thioridazine, and chlorpromazine than is the wild-type AG100, and this increased susceptibility is lost after it is induced to tetracycline resistance (Table 3). Overall, the response of the Δ*acrAB* tetracycline-induced resistant strain ($AG100A_{TET}$) to antibiotics, substrates, and some of the inhibitors of efflux pumps is now similar to those of the wild-type AG100 strain. If the marked susceptibility of the AG100A strain to tetracycline is only related to the deletion of the *acrAB* genes, then known inhibitors of efflux pumps at concentrations proven not to affect growth should render the *acrAB*-intact AG100 strain as susceptible to tetracycline as the mutant. Of the inhibitors of efflux pumps tested (identified in Table 3), only CCCP and PAN reduced the MIC of tetracycline against AG100 from 2.0 to 0.5 mg/liter. In addition, PAN reduced the susceptibility of AG100 to erythromycin from 100 mg/liter to 3.13 mg/liter, nalidixic acid from 5 mg/liter to 2.5 mg/liter, oxacillin from 256 mg/liter to 64 mg/liter, chloramphenicol from 8 mg/liter to 2 mg/liter, and rhodamine from

>800 mg/liter to 100 mg/liter. With respect to the effects of the above inhibitors on the susceptibility of the tetracycline-induced wild-type strain to tetracycline ($AG100_{TET}$), only PAN reduced the susceptibility of this strain to that of its parental noninduced strain, i.e., from 12 to 0.5 mg/liter. None of the agents employed in this study altered the resistance to tetracycline of the Δ*acrAB* mutant tetracycline-adapted strain ($AG100A_{TET}$).

In conclusion, both the wild-type *E. coli* AG100 and its *acrAB*-deleted mutant progeny, the latter far more sensitive to tetracycline, have the capacity to become increasingly resistant to tetracycline when exposure to tetracycline is gradually increased. The mechanism by which increased tolerance to tetracycline is brought about does not involve the selection of a mutation inasmuch as reversal to original tetracycline susceptibility could be obtained for both induced strains with their subsequent serial transfer to drug-free medium. The evaluation of genes that code for the inner-membrane transporter component of the nine efflux pumps studied shows that the deletion of the major AcrAB efflux pump is not essential for survival of *E. coli* as suggested by others (10) and is especially shown by the current study when the organism is grown under persistent tetracycline pressure. Because the *acrF* gene is markedly expressed by the *acrAB*-deleted mutant when continuously exposed to tetracycline, we conclude that the functions of the deleted AcrAB efflux pump are taken over by the AcrEF pump. Moreover, because PAN increases the sensitivity of the noninduced *acrAB* (AG100) and the tetracycline-induced resistant *acrAB* ($AG100_{TET}$) whereas it has no effect on the *acrAB*-deleted mutant induced to the same level of tetracycline resistance ($AG100A_{TET}$), we conclude that PAN is a specific inhibitor of the AcrAB pump and has no effect on the remaining efflux pumps that bestow resistance to tetracycline.

The approaches employed in this study reveal that efflux pumps play an important role in intrinsic resistance of *E. coli* to tetracycline—whose current chemotherapeutic use is limited due to the emergence of resistance (4, 5)—and that this intrinsic resistance can be increased by the overexpression and interplay of the different efflux pump genes present in the genome. The redundancy of at least nine efflux pumps, each one expressed to greater levels of activity when the organism is under antibiotic pressure, coupled to the demonstration that a single agent such as PAN inhibits only the AcrAB efflux pump, suggests that it may be rather difficult to find one agent that has the capacity to inhibit all of the efflux pumps of *E. coli* that bestow antibiotic resistance to the organism.

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